



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/DK96/00498 <b>(22) International Filing Date:</b> 29 November 1996 (29.11.96) <b>(30) Priority Data:</b> 1356/95 30 November 1995 (30.11.95) DK <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> AASLYNG, Dorrit [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). SØRENSEN, Niels, Henrik [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). RØRBÆK, Karen [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). <b>(74) Common Representative:</b> NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> AN ENZYME FOR DYING KERATINOUS FIBRES  <b>(57) Abstract</b>  The present invention relates to a dyeing composition, a method for dyeing keratinous fibres, in particular hair, fur, hide and wool, and the use of a <i>Scytalidium</i> laccase for dyeing.		

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**Title:** An enzyme for dying keratinous fibres

## 5 **FIELD OF THE INVENTION**

The present invention relates to a dyeing composition for keratinous fibres, in particular hair, fur, hide and wool, a method for dying and the use of a *Scytalidium* laccase for dyeing.

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## **BACKGROUND OF THE INVENTION**

It has been used for many years to dye the hair to cover appearing grey hair. The need to do so arises from the fact that grey hair is the first sign of having past adolescence, which can be hard to accept for many people.

For instance, in certain parts of Asia it is widely used by both men and women to dye the hair with dyes often referred to by humorous people as "black shoe polish".

During the last few decades hair dyeing has become more and more popular in the western world. At first Punk Rockers and other society critical groups dyed their hair in extreme colours as a part of their protest against the established society, but today especially many young people also uses hair dyes (in more soft tints than the Punk Rockers) as a sort of "cosmetic" to change or freshen up their "look".

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### **Hair dyes**

In general hair dyeing compositions on the market today can be divided into three main groups:

- 30 - temporary hair dyes,
- semi-permanent hair dyes, and
- permanent oxidative hair dyes.

The temporary hair dyes are only intended to change the natural hair colour for a short period of time and usually functions by depositing dyes on the surface of the hair. Such hair dyes are easy to remove with normal shampooing.

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When using semi-permanent hair dyes the colour of the dyed hair can survive for five or more shampoos. This is achieved

by using dyes having a high affinity for hair keratin and which is able to penetrate into the interior of the hair shaft.

Permanent hair dyes are very durable to sunlight, shampooing and other hair treatments and need only to be refreshed once a month as new hair grows out. With these dyeing systems the dyes are created directly in and on the hair. Small aromatic colourless dye precursors (e.g. p-phenylene-diamine and o-aminophenol) penetrate deep into the hair where said dye precursors are oxidised by an oxidising agent into coloured polymeric compounds. These coloured compounds are larger than the dye precursors and can not be washed out of the hair.

By including compounds referred to as modifiers (or couplers) in the hair dyeing composition a number of hair colour tints can be obtained. Cathecol and Resorcinol are examples of such modifiers.

Traditionally  $H_2O_2$  is used as the oxidizing agent (colour builder), but also as a bleaching agent. Dyeing compositions comprising  $H_2O_2$  are often referred to as "lightening dyes" due to this lightening effect of  $H_2O_2$ .

The use of  $H_2O_2$  in dye compositions have some disadvantages as  $H_2O_2$  damages the hair. Further, oxidative dyeing often demands high pH (normally around pH 9-10), which also inflicts damage on the hair. Consequently, if using dye compositions comprising  $H_2O_2$  it is not recommendable to dye the hair often.

To overcome the disadvantages of using  $H_2O_2$  it has been suggested to use oxidation enzymes to replace  $H_2O_2$ .

US patent no. 3,251,742 (Revlon) describes a method for dyeing human hair by dye formation *in situ* (i.e. on the hair). An oxidative enzyme is used to the colour formation reactions at a substantially neutral pH (pH 7-8.5).

Laccases, tyrosinases, polyphenolases and catacolases are mentioned as the suitable oxidation enzymes.

EP patent no. 504.005 (Perma S.A.) concerns compositions for dying hair which do not require the presence of  $H_2O_2$  (hydrogen peroxide). The composition comprises an enzyme capable of catalyzing the formation of the polymeric dyes and also dye precursors, such as bases and couplers, in a buffer solution wherein the pH of said composition is between 6.5 and 8 and

said enzyme has an optimal activity in the pH range between 6.5 and 8.

*Rhizoctonia praticola* laccase and *Rhus vernicifera* laccase have a pH-optimum between 6.5 and 8 and can be used to form the  
5 polymeric dyes according to this patent.

Abstract of Papers American Chemical Society vol. 209, no. 1-2, 1995 discloses the cloning of a laccase from a *Scytalidium thermophilum*. The abstract does not mention the use of said laccase for dyeing hair.

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#### SUMMARY OF THE INVENTION

The object of the present invention is to provide improved permanent dyeing compositions for keratinous fibres, in particular hair, fur, hide and wool, which is less damaging to the  
15 keratinous fibres than e.g. dyeing compositions for hair using  $H_2O_2$ .

It has now surprisingly been found that it is possible to provide such an improved dyeing composition by using an enzyme derived from a strain of the filamentous fungus genus *Scytalidium* as the oxidation enzyme.  
20

In the first aspect the invention relates to a permanent dyeing composition for keratinous fibres, in particular hair, fur, hide and wool, comprising an oxidation enzyme comprising  
1) one or more oxidation enzymes derived from a strain of the  
25 genus *Scytalidium*,  
2) one or more dye precursors, and  
optionally 3) one or more modifiers.

In a preferred embodiment of the invention the oxidation enzyme is a laccase derived from a strain of the genus *Scytalidium*,  
30 *in particular from a strain of the species Scytalidium thermophilum*.

Secondly, it is the object of the invention to provide a method for dying keratinous fibres, comprising contacting a laccase derived from a strain of the genus *Scytalidium* with the  
35 keratinous fibres and at least one dye precursor in the presence or absence of at least one modifier for a suitable period of time and under conditions sufficient to permit oxidation of the dye precursor into a coloured compound.

Finally the invention relates to the use of an oxidation enzyme derived from a strain of the genus *Scytalidium* for oxidative dyeing of keratinous fibres, in particular hair, fur, hide and wool.

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#### BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows the dyeing effect of the *Scytalidium thermophilum* laccase (rStL-FXu-1)

#### 10 DETAILED DESCRIPTION OF THE INVENTION

The object of the present invention is to provide improved permanent dyeing compositions for keratinous fibres, in particular hair, fur, hide and wool, which is less damaging to the keratinous fibres than e.g. hair dyeing compositions using  $H_2O_2$ .

15 It has surprisingly be found that it is possible to provide such an improved dyeing composition by using an oxidation enzyme derived from a strain of the filamentous fungus genus *Scytalidium*.

When using said oxidation enzyme derived from a strain of the genus *Scytalidium* the colour developed is as wash stable as oxidative dyeing of e.g. hair using  $H_2O_2$  and the light fastness is as good as when dyeing chemically.

Consequently, in the first aspect the present invention relates to a permanent dye composition for keratinous fibres , in particular hair, fur, hide and wool, comprising

- 25 1) one or more oxidation enzymes derived from a strain of the genus *Scytalidium*,  
2) one or more dye precursors, and  
optionally 3) one or more modifiers.

30 In an embodiment of the invention the oxidation enzyme is a laccase derived from a strain of genus *Scytalidium*, such as a strain of *Scytalidium thermophilum* e.g. the purified laccase described in WO 95/33837 (PCT/US95/06816) from Novo Nordisk, which is hereby incorporated. SEQ ID No 1 shows a DNA sequence  
35 encoding a suitable laccase derivable from a strain of the species *Scytalidium thermophilum*.

*E. coli* JM101 containing the expression vector pShTh15 comprising SEQ ID NO 1 has been deposited under the Budapest

Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria, Illinois, 61604. The vector have been given the Accession Number NRRL B-21262.

- 5 Also contemplated according to the invention are laccases derived from other microorganisms being more than 80% homologous to SEQ ID NO 1 derived from a strain of the species *Scytalidium thermophilum*.

In addition, *Scytalidium* laccases also encompass alternative  
10 forms of laccases which may be found in *S. thermophilum* and as well as laccases which may be found in other fungi which are synonyms of fall within the definition of *S. thermophilum* as defined by Straatsma and Samson, (1993), Mycol. Res. 97, 321-328). These include *S. indonesiacum*, *Torula thermophila*, *Humicola brevis* var. *thermoidea*, *Humicola brevispora*, *H. grisea* var. *thermoidea*, *Humicola insolens*, and *Humicola lanuginosa*  
15 (also known as *Thermomyces lanuginosus*).

It is to be understood that the *Scytalidium* laccase may be produced homologously, or heterologously using filamentous  
20 fungus, yeast or bacteria as the host cell.

Examples of filamentous fungi host cells include strains of the species of *Trichoderma*, preferably a strain of *Trichoderma harzianum* or *Trichoderma reesei*, or a species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger*, or  
25 yeast cells, such as e.g. a strain of *Saccharomyces*, in particular *Saccharomyces cerevisiae*, *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such as *Schizosaccharomyces pombe*, a strain of *Hansenula* sp., *Pichia* sp., *Yarrowia* sp., such as *Yarrowia lipolytica*, or *Kluyveromyces* sp., such as *Kluyveromyces lactis*, or a bacteria, such as  
30 gram-positive bacteria such as strains of *Bacillus*, such as strains of *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus*, *B. megaterium* or *B. thuringiensis*, or strains of *Streptomyces*, such as *S. lividans* or *S. murinus*, or gram-negative bacteria such as *Escherichia coli*.  
35

Laccases (benzenediol:oxygen oxidoreductases) (E.C. class

1.10.3.2 according to Enzyme Nomenclature (1992) Academic Press, Inc) are multi-copper containing enzymes that catalyze the oxidation of phenols. Laccase-mediated oxidations result in the production of aryloxy-radical intermediates from suitable phenolic substrates; the ultimate coupling of the intermediates so produced provides a combination of dimeric, oligomeric, and polymeric reaction products. Certain reaction products can be used to form dyes suitable for dyeing hair (see below).

In an embodiment of the invention the *Scytalidium* laccase is neutral. In the context of laccases of the present invention this means that the pH optimum lies in the range from between 6.0 and 8.0.

To obtain dyeing of the keratinous fibres, such as hair, the dyeing composition of the invention also comprises a dye precursor which is converted into a coloured compound (i.e. a dye) by the oxidation agent which according to the invention is an oxidation enzyme derived from a strain of the species *Scytalidium*, such as a strain of *Scytalidium thermophilum*.

Without being limited thereto the dye precursor(s) may be (an) aromatic compound(s) belonging to one of three major chemical families: the diamines, aminophenols (or aminonaphtols) and the phenols. Examples of isatin derivative dye precursors can be found in DE 4,314,317-A1. Further, a number of indole or indoline derivative dye precursors are disclosed in WO 94/00100. Said dye precursors mentioned in these documents are hereby incorporated herein by reference.

Examples of such suitable dye precursors include compounds from the group comprising p-phenylene-diamine (PPD), p-tolylene-diamine, chloro-p-phenylenediamine, p-aminophenol, o-aminophenol and 3,4-diaminotoluene, 2-methyl-1,4-diaminobenzene, 4-methyl-o-phenylenediamine, 2-methoxy-p-phenylenediamine, 2-chloro-1,4-diamino-benzene, 4-amino diphenylamine, 1-amino-4- $\beta$ -methoxyethylamino-benzene, 1-amino-4-bis-( $\beta$ -hydroxyethyl)-aminobenzene, 1-3-diamino-benzene, 2-methyl-1,3-diamino-benzene, 2,4-diaminotoluene, 2,6-diaminopyridine, 1-hydroxy-2-amino-benzene, 1-hydroxy-3-amino-benzene, 1-methyl-2-hydroxy-4-amino-benzene, 1-methyl-2-hydroxy-4- $\beta$ -hydroxyethylamino-benzene, 1-



hydroxy-4-amino-ebnzene, 1-hydroxy-4-methylamino-benzene, 1-methoxy-2,4-diamino-benzene, 1-ethoxy-2,3-diamino-benzene, 1- $\beta$ -hydroxyethyloxy-2,4-diamino-benzene, phenazines, such as 4,7-phenazinedicarboxylic acid, 2,7-phenazinedicarboxylic acid, 2-phenazinecarboxylic acid, 2,7-diaminophenazine, 2,8-diaminophenazine, 2,7-diamino-3,8-dimethoxyphenazine, 2,7-diamino-3-methoxyphenazine, 2,7-diamino 3-methoxyphenazine, 3-dimethyl 2,8-phenazinediamine, 2,2'-[(8-amino-7-methyl-2-phenazinyl)imino]bis-ethanol, 2,2'-[(8-amino-7-methoxy-2-phenazinyl)imino]bis-ethanol, 2,2'-[(8-amino-7-chloro-2-phenazinyl)imino]bis-ethanol, 2-[(8-amino-7-methyl-2-phenazinyl)amino]-ethanol, 2,2'-[(8-amino-2-phenazinyl)imino]bis-ethanol, 3-amino-7-(dimethylamino)-2,8-dimethyl-5-phenyl-chloride, 9-(diethylamino)-benzo[a]phenazine-1,5-diol, N-[8-(diethylamino)-2-phenazinyl]-methanesulfonamide, N-(8-methoxy-2-phenazinyl)-Methanesulfonamide, N,N,N',N'-tetramethyl-2,7-phenazinediamine, 3,7-dimethyl-2-phenazinamine, p-amino benzoic acids, such as p-amino benzoic acid ethyl, p-amino benzoic acid glycerid, p-amino benzoic acid isobutyl, p-dimethylamino benzoic acid amil, p-dimethylamino benzoic acid octyl, p-diethoxy amino benzoic acid amil, p- dipropoxy amino benzoic acid ethyl, acetylsalicylic acid, isatin derivatives, such as 2,3-diamino benzoic acid.

In an embodiment the laccase is used with the dye precursor directly to oxidise it into a coloured compound. The dye precursor may be used alone or in combination with other dye precursors.

However, it is believed that when using a diamine or an aminophenol as the dye precursor at least one of the intermediate in the copolymerisation must be an *ortho*- or *para*-diamine or aminophenol. Examples of such are described in US patent no. 3,251,742 (Revlon), the contents of which are incorporated herein by reference.

Optionally the dyeing composition of the invention (especially hair dyeing compositions) also comprises a modifier (coupler) by which a number of colour tints can be obtained. In general modifiers are used in hair dyeing compositions, as the colours resulting from hair dyeing compositions without modifier(s) are usually unacceptable for most people.

Modifiers are typically m-diamines, m-aminophenols, or polyphenols. The modifier (coupler) reacts with the dye precursor(s) in the presence of the oxidative enzyme, converting it into a coloured compound.

5        Examples of modifiers (couplers) include m-phenylenediamine, 2,4-diaminoanisole, 1-hydroxynaphthalene ( $\alpha$ -naphthol), 1,4-dihydroxybenzene (hydroquinone), 1,5-dihydroxynaphthalene, 1,2-dihydroxybenzene (pyrocatechol), 1,3-dihydroxybenzene (resorcinol), 1,3-dihydroxy-2-methylbenzene, 1,3-dihydroxy-4-chlorobenzene (4-chlororesorcinol), 1,2,3-trihydroxybenzene, 10        1,2,4-trihydroxybenzene, 1,2,4-trihydroxy-5-methylbenzene, 1,2,4-trihydroxytoluene.

In the second aspect the invention relates to a method for dyeing keratinous fibres, in particular hair, fur, hide and 15        wool, comprising contacting a laccase derived from a strain of the genus *Scytalidium* with the keratinous fibres and at least one dye precursor in the presence or absence of at least one modifier, for a period of time and under conditions sufficient to permit oxidation of the dye precursor into coloured 20        compounds (i.e. a dye).

The dyeing method can be conducted with one or more dye precursors, either alone or in combination with one or more modifiers.

The amount of dye precursor(s) and other ingredients used in 25        the composition of the invention are in accordance with usual commercial amounts.

When using a *Scytalidium* laccase, such as the *Scytalidium thermophilum* laccase mentioned above, the method for dyeing keratinous fibres of the invention may be carried out at room 30        temperature, preferably around the optimum temperature of the enzyme, at a pH in the range from 3.0 to 9.0, preferably 4.0 to 8.0, especially pH 6.0 to 8.0.

Suitable dye precursors and optional modifiers are described above.

35        The use of this *Scytalidium* laccase is an improvement over the more traditional use of  $H_2O_2$  as the latter can damage the keratinous fibres, such as hair. Further, normally prior art methods requires a high pH, which is also damaging to the

keratinous fibres. In contrast hereto, the reaction with laccase can be conducted at acidic or neutral pH, and the oxygen needed for oxidation comes from the air, rather than via harsh chemical oxidation.

5 The result provided by the use of the *Scytalidium* laccase is comparable to that achieved with use of  $H_2O_2$ , not only in colour development, but also in wash stability and light fastness. An additional commercial advantage is that a single container package can be made containing both the laccase and the precursor, in an oxygen free atmosphere, which arrangement is not possible with the use of  $H_2O_2$ .

#### MATERIALS AND METHODS

##### Materials:

##### 15 Hair:

6" De Meo Virgin Natural White Hair (De Meo Brothers Inc. US)

##### Enzymes:

Laccase from *Scytalidium thermophilum* described in

20 WO 95/33837 (PCT/US95/06816) from Novo Nordisk

##### Deposit of Biological Material

The following biological material has been deposited on the 25<sup>th</sup> May 1994 under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria, Illinois, 61604 and given the following accession number.

30	Deposit	Accession Number
	<i>E. coli</i> JM101 containing pShTh15	NRRL B-21262.

##### Dye precursors:

0.1 % w/w p-phenylene-diamine in 0.1 M K-phosphate buffer, pH 7.0. (pPD)

0.1 % w/w p-toluylene-diamine in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % w/w chloro-p-phenylenediamine in 0.1 M K-phosphate

buffer, pH 7.0.

0.1 % w/w p-aminophenol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % w/w o-aminophenol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % w/w 3,4-diaminotoluene in 0.1 M K-phosphate, buffer pH  
5 7.0.

Modifiers:

0.1 % w/w m-phenylene-diamine in 0.1 M K-phosphate buffer, pH  
7.0.

10 0.1 % w/w 2,4-diaminoanisole in 0.1 M K-phosphate buffer, pH  
7.0.

0.1 % w/w a-naphthol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % w/w hydroquinone in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % w/w pyrocatechol in 0.1 M K-phosphate buffer, pH 7.0.

15 0.1% w/w resorcinol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % w/w 4-chlororesorcinol in 0.1 M K-phosphate buffer, pH  
7.0.

The dye precursor is combined with one of the above  
indicated modifiers so that the final concentration in the  
20 dyeing solution is 0.1 % w/w with respect to precursor and 0.1  
% w/w with respect to modifier.

Other solutions:

3% H<sub>2</sub>O<sub>2</sub> (in the final dye solution)

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Commercial shampoo

Equipment:

Minolta CR200 Chroma Meter

30 Day light bulb: 1000 LUX (D65)

Determination of Laccase Activity (LACU)

Laccase activity is determined from the oxidation of syring-  
galdazin under aerobic conditions. The violet colour produced  
35 is photometered at 530 nm. The analytical conditions are 19 mM  
syringaldazin, 23.2 mM acetate buffer, pH 5.5, 30°C, 1 min.  
reaction time.

1 laccase unit (LACU) is the amount of enzyme that catalyses

the conversion of 1.0 micromole syringaldazin per minute at these conditions.

### Assessment of the hair colour

- 5       The quantitative colour of the hair tresses are determined on a Minolta CR200 Chroma Meter by the use the parameters  $L^*$  ("0"=black and "100"=white),  $a^*$  ("-"=green and "+"=red) and  $b^*$  ("-" blue and "+" yellow).
- 10    $DL^*$ ,  $Da^*$  and  $Db^*$  are the delta values of  $L^*$ ,  $a^*$  and  $b^*$  respectively compared to  $L^*$ ,  $a^*$  and  $b^*$  of untreated hair (e.g.  $DL^* = L^*_{\text{sample}} - L^*_{\text{untreated hair}}$ ).

$DE^*$  is calculated as  $DE^* = \sqrt{DL^{*2} + Da^{*2} + Db^{*2}}$  and is an expression  
15   for the total quantitative colour change.

### EXAMPLES

#### Example 1

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#### Dyeing effect

The dyeing effect of a *Scytalidium thermophilum* laccase was tested using the dye precursor o-aminophenol and the modifier m-phenylenediamine.

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#### Hair dyeing

1 gram De Meo white hair tresses were used.

- 4 ml dye precursor solution (including modifier) is mixed with 1 ml laccase on a Whirley mixer, applied to the hair  
30   tresses and incubated at 30°C for 60 minutes.

The hair tresses are then rinsed with running water, washed with shampoo, rinsed with running water, combed, and air dried.

The  $a^*$ ,  $b^*$  and  $L^*$  was determined on the Chroma Meter and the  $DE^*$  values were then calculated.

- 35   A hair tress sample treated without enzyme was used as a blind.

The result of the hair dyeing test is shown in figure 1.

**Example 2**Wash stability

Tresses of white De Meo hair (1 gram) is used for testing the wash stability of hair dyed using *Scytalidium thermophilum* laccase, compared with hair dyed using  $H_2O_2$ , and p-phenylenediamine (pPD) as the dye precursor. Further the wash stability is compared with a commercial oxidative dye.

The oxidative hair dyeing is carried out as described in Example 1.

Hair wash

The dyed hair tresses are wetted and washed for 15 seconds with 50 ml of commercial shampoo, and rinsed with water for 1 minute and air dried. The hair tresses are washed up to 18 times.

The  $a^*$ ,  $b^*$  and  $L^*$  is determined on the Chroma Meter and the  $\Delta E^*$  values are then calculated.

**Example 3**The light fastness

Tresses of blond European hair are used for testing the light fastness of hair dyed using *Scytalidium thermophilum* laccase in comparison to hair dyed using  $H_2O_2$ . p-phenylenediamine was used as dye precursor.

The dyeing of the hair was carried out as described in Example 1.

One hair tress is kept dark, while an other is kept at day light (i.e. under a day light bulb (D65)), at approximately 1000 LUX) for up to 275 hours.

The  $a^*$ ,  $b^*$  and  $L^*$  parameters are determined immediately after the dyeing of the hair, and further during exposure to day light.

$DE^*$  then calculated from the determined  $a^*$ ,  $b^*$  and  $L^*$  values.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## 5 (i) APPLICANT:

(A) NAME: Novo Nordisk A/S  
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(ii) TITLE OF INVENTION: An enzyme for dying hair

15 (iii) NUMBER OF SEQUENCES: 2

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
 (B) COMPUTER: IBM PC compatible  
 20 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## 25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2476 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

35 (A) ORGANISM: *Scytalidium thermophilum*

## (ix) FEATURE:

(A) NAME/KEY: intron  
 (B) LOCATION: 349..411

## 40 (ix) FEATURE:

(A) NAME/KEY: intron  
 (B) LOCATION: 502..559

## 45 (ix) FEATURE:

(A) NAME/KEY: intron  
 (B) LOCATION: 632..686

## (ix) FEATURE:

50 (A) NAME/KEY: intron  
(B) LOCATION: 1739..1804

## (ix) FEATURE:

55 (A) NAME/KEY: CDS  
(B) LOCATION: join (106..348, 412..501, 560..631, 687..1738, 1805..2194)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CTGAATTAA ATACAGGAAG ATCGCATTCA ATCCAGCCTA GACTGCACAA TGGTTCTGCA 60  
 60 CGACCGTCGC ACACCTGCCA ATAGTGTTAA TAACGGNCTA ATACC ATG AAG CGC TTC 117  
 Met Lys Arg Phe  
 1  
 65 TTC ATT AAT AGC CTT CTG CTT CTC GCA GGG CTC CTC AAC TCA GGG GCC 165  
 Phe Ile Asn Ser Leu Leu Leu Ala Gly Leu Leu Asn Ser Gly Ala  
 5 10 15 20  
 CTC GCG GCT CCG TCT ACA CAT CCC AGA TCA AAC CCC GAC ATA CTG CTT 213

	Leu	Ala	Ala	Pro	Ser	Thr	His	Pro	Arg	Ser	Asn	Pro	Asp	Ile	Leu	Leu	
				25						30					35		
5	GAA	AGA	GAT	GAC	CAC	TCC	CTT	ACG	TCT	CGG	CAA	GGT	AGC	TGT	CAT	TCT	261
	Glu	Arg	Asp	Asp	His	Ser	Leu	Thr	Ser	Arg	Gln	Gly	Ser	Cys	His	Ser	
				40					45					50			
10	CCA	AGC	AAC	CGC	GCC	TGT	TGG	TGC	TCT	GGC	TTC	GAT	ATC	AAC	ACG	GAT	309
	Pro	Ser	Asn	Arg	Ala	Cys	Trp	Cys	Ser	Gly	Phe	Asp	Ile	Asn	Thr	Asp	
			55					60					65				
15	TAT	GAG	ACC	AAG	ACT	CCA	AAC	ACC	GGA	GTG	GTG	CGG	CGG	GTTAGTATCC			358
	Tyr	Glu	Thr	Lys	Thr	Pro	Asn	Thr	Gly	Val	Val	Arg	Arg				
		70					75					80					
20	CAAGTTACGT	TTGACCAAGA	AATGGACGTG	AAGTGTGCTG	ACTCTCCCGC	TAG											411
	TAC	ACC	TTT	GAT	ATC	ACC	GAA	GTC	GAC	AAC	CGC	CCC	GGT	CCC	GAT	GGG	459
	Tyr	Thr	Phe	Asp	Ile	Thr	Glu	Val	Asp	Asn	Arg	Pro	Gly	Pro	Asp	Gly	
				85					90					95			
25	GTC	ATC	AAG	GAG	AAG	CTC	ATG	CTT	ATC	AAC	GAC	AAA	CTC	CTG	GTAGG		506
	Val	Ile	Lys	Glu	Lys	Leu	Met	Leu	Ile	Asn	Asp	Lys	Leu	Leu			
			100					105					110				
30	GTCCTCTCGA	ACGCCTGCGT	CTGCCACACA	GCGTAAACT	AACGAACCGC	TAG											559
	GGC	CCG	ACA	GTC	TTC	GCA	AAC	TGG	GGC	GAC	ACC	ATC	GAG	GTG	ACC	GTC	607
	Gly	Pro	Thr	Val	Phe	Ala	Asn	Trp	Gly	Asp	Thr	Ile	Glu	Val	Thr	Val	
				115					120					125			
35	AAC	AAC	CAC	CTG	AGA	ACC	AAC	GGA	GTAAGCGTTC	GGACACAAAG	CCCAGCAACC						661
	Asn	Asn	His	Leu	Arg	Thr	Asn	Gly									
			130					135									
40	TAGACACACT	CAACTGACCA	AGTAG	ACC	TCC	ATC	CAC	TGG	CAC	GGC	TTG	CAC	CAA				716
				Thr	Ser	Ile	His	Trp	His	Gly	Leu	His	Gln				145
								140									
45	AAA	GGA	ACC	AAC	TAC	CAC	GAC	GGC	GCC	AAC	GGC	GTG	ACC	GAG	TGT	CCC	764
	Lys	Gly	Thr	Asn	Tyr	His	Asp	Gly	Ala	Asn	Gly	Val	Thr	Glu	Cys	Pro	
				150						155					160		
50	ATC	CCG	CCC	GGT	GGC	TCC	CGA	GTC	TAC	AGC	TTC	CGA	GCG	CGC	CAA	TAT	812
	Ile	Pro	Pro	Gly	Gly	Ser	Arg	Val	Tyr	Ser	Phe	Arg	Ala	Arg	Gln	Tyr	
				165				170						175			
55	GGA	ACG	TCA	TGG	TAC	CAC	TCC	CAC	TTC	TCC	GCC	CAG	TAT	GGC	AAC	GGC	860
	Gly	Thr	Ser	Trp	Tyr	His	Ser	His	Phe	Ser	Ala	Gln	Tyr	Gly	Asn	Gly	
			180					185					190				
60	GTG	AGC	GGC	GCC	ATC	CAG	ATC	AAC	GGA	CCC	GCC	TCC	CTG	CCC	TAC	GAC	908
	Val	Ser	Gly	Ala	Ile	Gln	Ile	Asn	Gly	Pro	Ala	Ser	Leu	Pro	Tyr	Asp	
		195					200					205					
65	ATC	GAC	CTC	GGC	GTC	CTC	CCG	CTG	CAG	GAC	TGG	TAC	TAC	AAG	TCC	GCC	956
	Ile	Asp	Leu	Gly	Val	Leu	Pro	Leu	Xaa	Asp	Trp	Tyr	Tyr	Lys	Ser	Ala	
		210				215					220					225	
70	GAC	CAG	CTC	GTC	ATC	GAG	ACC	CTG	GCC	AAG	GGC	AAC	GCT	CCG	TTC	AGC	1004
	Asp	Gln	Leu	Val	Ile	Glu	Thr	Leu	Xaa	Lys	Gly	Asn	Ala	Pro	Phe	Ser	
					230				235						240		
75	GAC	AAC	GTC	CTC	ATC	AAC	GGC	ACC	GCA	AAG	CAC	CCC	ACC	ACT	GGC	GAA	1052
	Asp	Asn	Val	Leu	Ile	Asn	Gly	Thr	Ala	Lys	His	Pro	Thr	Thr	Gly	Glu	
				245					250					255			
80	GGG	GAG	TAC	GCC	ATC	GTG	AAG	CTC	ACC	CCG	GGC	AAA	CGC	CAT	CGC	CTG	1100
	Gly	Glu	Tyr	Ala	Ile	Val	Lys	Leu	Thr	Pro	Asp	Lys	Arg	His	Arg	Leu	



	260					265					270								
5	CGG Arg	CTC Leu	ATC Ile	AAC Asn	ATG Met	TCG Ser	GTG Val	GAG Glu	AAC Asn	CAC His	TTC Phe	CAG Gln	GTC Val	TCG Ser	CTG Leu	GCG Ala	1148		
	275						280					285							
10	AAG Lys	CAC His	ACC Thr	ATG Met	ACG Thr	GTC Val	ATC Ile	GCG Ala	GCG Ala	GAC Asp	ATG Met	GTC Val	CCC Pro	GTC Val	AAC Asn	GCC Ala	1196		
	290					295					300					305			
15	ATG Met	ACC Thr	GTC Val	GAC Asp	AGC Ser	CTG Leu	TTT Phe	ATG Met	GCC Ala	GNC Val	GGG Gly	CAG Gln	CGG Arg	TAT Tyr	GAT Asp	GTT Val	1244		
					310					315					320				
20	ACC Thr	ATC Ile	GAC Asp	GCG Ala	AGC Ser	CAG Gln	GCG Ala	GTG Val	GGG Gly	AAT Asn	TAC Tyr	TGG Trp	TTC Phe	AAC Asn	ATC Ile	ACC Thr	1292		
				325					330					335					
25	TTT Phe	GGA Gly	GGG Gly	CAG Gln	CAG Gln	AAG Lys	TGC Cys	GGC Gly	TTC Phe	TCG Ser	CAC His	AAT Asn	CCG Pro	GCG Ala	CCG Pro	GCA Ala	1340		
		340						345					350						
30	GCC Ala	ATC Ile	TTT Phe	CGC Arg	TAC Tyr	GAG Glu	GGC Gly	GCT Ala	CCT Pro	GAC Asp	GCT Ala	CTG Leu	CCG Pro	ACG Thr	GAT Asp	CCT Pro	1388		
		355					360					365							
35	GGC Gly	GCT Ala	GCG Ala	CCA Pro	AAG Lys	GAT Asp	CAT His	CAG Gln	TGC Cys	CTG Leu	GAC Asp	ACT Thr	TTG Leu	GAT Asp	CTT Leu	TCA Ser	1436		
	370					375					380					385			
40	CCG Pro	GTG Val	GTG Val	CAA Gln	AAG Lys	AAC Asn	GTG Val	CCG Pro	GTT Val	GAC Asp	GGG Gly	TTC Phe	GTC Val	AAA Lys	GAG Glu	CCT Pro	1484		
					390					395					400				
45	GGC Gly	AAT Asn	ACG Thr	CTG Leu	CCG Pro	GTG Val	ACG Thr	CTC Leu	CAT His	GTT Val	GAC Asp	CAG Gln	GCC Ala	GCG Ala	GCT Ala	CCA Pro	1532		
				405					410					415					
50	CAC His	GTG Val	TTT Phe	ACG Thr	TGG Trp	AAG Lys	ATC Ile	AAC Asn	GGG Gly	AGC Ser	GCT Ala	GCG Ala	GAC Asp	GTG Val	GAC Asp	TGG Trp	1580		
			420					425					430						
55	GAC Asp	AGG Arg	CCG Pro	GTG Val	CTG Leu	GAG Glu	TAT Tyr	GTC Val	ATG Met	AAC Asn	AAT Asn	GAC Asp	CTG Leu	TCT Ser	AGC Ser	ATT Ile	1628		
		435					440					445							
60	CCG Pro	GTC Val	AAG Lys	AAC Asn	AAC Asn	ATT Ile	GTG Val	AGG Arg	GTG Val	GAC Asp	GGA Gly	GTC Val	AAC Asn	GAG Glu	TGG Trp	ACG Thr	1676		
	450					455					460					465			
65	TAC Tyr	TGG Trp	CTC Leu	GTC Val	GAA Glu	AAC Asn	GAC Asp	CCG Pro	GAG Glu	GGC Gly	CGC Arg	CTC Leu	AGT Ser	TTG Leu	CCG Pro	CAT His	1724		
					470					475					470				
70	CCG Pro	ATG Met	CAT His	CTA Leu	CAC His	GTAAGTCACA					TCCCCACTA		CCATTCGGAA		TGACCACCAG		1779		
				475															
75	GTACTGACAC					CCTCCTCCTC		AATAG		GGA Gly	CAC His	GAT Asp	TTC Phe	TTT Phe	GTC Val	CTA Leu	GGC Gly	CGC Arg	1831
												480						485	
80	TCC Ser	CCC Pro	GAC Asp	GTC Val	TCG Ser	CCC Pro	GAT Asp	TCA Ser	GAA Glu	ACC Thr	CGC Arg	TTC Phe	GTC Val	TTT Phe	GAC Asp	CCG Pro	1879		
					490					495					500				
85	GCC Ala	GTC Val	GAC Asp	CTC Leu	CCC Pro	CGT Arg	CTG Leu	CGC Arg	GGA Gly	CAC His	AAC Asn	CCC Pro	GTC Val	CGG Arg	CGC Arg	GAC Asp	1927		
				505					510					515					

5 GTC ACC ATG CTT CCC GCG CGC GGC TGG CTG CTG CTG GCC TTC CGC ACG 1975  
Val Thr Met Leu Pro Ala Arg Glu Trp Leu Leu Leu Ala Phe Arg Thr  
520 525 530

5 GAC AAC CCG GGC GCG TGG TTG TTC CAC TGC CAC ATC GCG TGR CAC GTG 2023  
Asp Asn Pro Gly Ala Trp Leu Phe His Cys His Ile Ala Trp His Val  
535 540 545

10 TCG GGC GGG TTA AGC GTC GAC TTT CTG GAG CGG CCG GAC GAG CTG CGC 2071  
Ser Gly Gly Leu Ser Val Asp Phe Leu Glu Arg Pro Asp Glu Leu Arg  
550 555 560 565

15 GGG CAG CTG ACG GGA GAG AGC AAG GCG GAG TTG GAG CGT GTT TGT CGC 2119  
Gly Gln Leu Thr Gly Glu Ser Lys Ala Glu Leu Glu Arg Val Cys Arg  
570 575 580

20 GAG TGG AAG GAT TGG GAG GCG AAG AGC CCG CAT GGG AAG ATC GAT TCG 2167  
Glu Trp Lys Asp Trp Glu Ala Lys Ser Pro His Gly Lys Ile Asp Ser  
585 590 595

25 GGG TTG AAG CAG CGG CGA TGG GAT GCG TGAGGTAGTT GGGCGGATTG 2214  
Gly Leu Lys Gln Arg Arg Trp Asp Ala  
600 605

25 TTTAACACGT AGTGGGTAAG GTTGGGGCGG GTTTGTTTGG CGTTTTTCAGG GGTGTTGGGGTG 2274

CGGATGCTGG TCATCCGGGA AACGGCTCTA CAACTGGTGT CAATAGACTA ATATAGAGTG 2334

30 ATCAAAGAAC TGAGGTTCTG AAAGAGGCGT GGAAGTCGCG TTGTGACTCC CTTTGCCATG 2394

TTGGGAAGTG TGGCTCAACA TTGTGTTTCTAG GTTTGCTCAG GGTGATNTCG AACTGACGTN 2454

35 TTGATGAGGG TTATTGCNTA GA 2476

(2) INFORMATION FOR SEQ ID NO: 2:

40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 616 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: *Scytalidium thermophilum*

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Lys Arg Phe Phe Ile Asn Ser Leu Leu Leu Leu Ala Gly Leu Leu  
1 5 10 15

55 Asn Ser Gly Ala Leu Ala Ala Pro Ser Thr His Pro Arg Ser Asn Pro  
20 25 30

Asp Ile Leu Leu Glu Arg Asp Asp His Ser Leu Thr Ser Arg Gln Gly  
35 40 45

60 Ser Cys His Ser Pro Ser Asn Arg Ala Cys Trp Cys Ser Gly Phe Asp  
50 55 60

65 Ile Asn Thr Asp Tyr Glu Thr Lys Thr Pro Asn Thr Gly Val Val Arg  
65 70 75 80

Arg Tyr Thr Phe Asp Ile Thr Glu Val Asp Asn Arg Pro Gly Pro Asp  
85 90 95

	Gly	Val	Ile	Lys	Glu	Lys	Leu	Met	Leu	Ile	Asn	Asp	Lys	Leu	Leu	Gly
				100					105					110		
5	Pro	Thr	Val	Phe	Ala	Asn	Trp	Gly	Asp	Thr	Ile	Glu	Val	Thr	Val	Asn
			115					120					125			
	Asn	His	Leu	Arg	Thr	Asn	Gly	Thr	Ser	Ile	His	Trp	His	Gly	Leu	His
		130					135					140				
10	Gln	Lys	Gly	Thr	Asn	Tyr	His	Asp	Gly	Ala	Asn	Gly	Val	Thr	Glu	Cys
	145					150					155					160
	Pro	Ile	Pro	Pro	Gly	Gly	Ser	Arg	Val	Tyr	Ser	Phe	Arg	Ala	Arg	Gln
					165					170					175	
15	Tyr	Gly	Thr	Ser	Trp	Tyr	His	Ser	His	Phe	Ser	Ala	Gln	Tyr	Gly	Asn
				180					185					190		
20	Gly	Val	Ser	Gly	Ala	Ile	Gln	Ile	Asn	Gly	Pro	Ala	Ser	Leu	Pro	Tyr
			195					200					205			
	Asp	Ile	Asp	Leu	Gly	Val	Leu	Pro	Leu	Gln	Asp	Trp	Tyr	Tyr	Lys	Ser
		210					215					220				
25	Ala	Asp	Gln	Leu	Val	Ile	Glu	Thr	Leu	Ala	Lys	Gly	Asn	Ala	Pro	Phe
	225					230					235					240
	Ser	Asp	Asn	Val	Leu	Ile	Asn	Gly	Thr	Ala	Lys	His	Pro	Thr	Thr	Gly
					245					250					255	
30	Glu	Gly	Glu	Tyr	Ala	Ile	Val	Lys	Leu	Thr	Pro	Asp	Lys	Arg	His	Arg
				260					265					270		
35	Leu	Arg	Leu	Ile	Asn	Met	Ser	Val	Glu	Asn	His	Phe	Gln	Val	Ser	Leu
			275					280					285			
	Ala	Lys	His	Thr	Met	Thr	Val	Ile	Ala	Ala	Asp	Met	Val	Pro	Val	Asn
		290					295					300				
40	Ala	Met	Thr	Val	Asp	Ser	Leu	Phe	Met	Ala	Xaa	Gly	Gln	Arg	Tyr	Asp
	305					310					315					320
	Val	Thr	Ile	Asp	Ala	Ser	Gln	Ala	Val	Gly	Asn	Tyr	Trp	Phe	Asn	Ile
					325					330					335	
45	Thr	Phe	Gly	Gly	Gln	Gln	Lys	Cys	Gly	Phe	Ser	His	Asn	Pro	Ala	Pro
				340					345					350		
50	Ala	Ala	Ile	Phe	Arg	Tyr	Glu	Gly	Ala	Pro	Asp	Ala	Leu	Pro	Thr	Asp
			355					360					365			
	Pro	Gly	Ala	Ala	Pro	Lys	Asp	His	Gln	Cys	Leu	Asp	Thr	Leu	Asp	Leu
		370					375					380				
55	Ser	Pro	Val	Val	Gln	Lys	Asn	Val	Pro	Val	Asp	Gly	Phe	Val	Lys	Glu
	385					390					395					400
	Pro	Gly	Asn	Thr	Leu	Pro	Val	Thr	Leu	His	Val	Asp	Gln	Ala	Ala	Ala
					405					410					415	
60	Pro	His	Val	Phe	Thr	Trp	Lys	Ile	Asn	Gly	Ser	Ala	Ala	Asp	Val	Asp
				420					425					430		
65	Trp	Asp	Arg	Pro	Val	Leu	Glu	Tyr	Val	Met	Asn	Asn	Asp	Leu	Ser	Ser
			435					440					445			
	Ile	Pro	Val	Lys	Asn	Asn	Ile	Val	Arg	Val	Asp	Gly	Val	Asn	Glu	Trp
		450					455					460				



## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13 bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>9</u> , line <u>21-31</u>	
B. IDENTIFICATION OF <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depository institution Agricultural Research Service Patent Culture Collection (NRRL)	
Address of depository institution (including postal code and country)  Northern Regional Research Center 1815 University Street Peoria, IL 61604, US	
Date of deposit 25 May 1994	Accession Number NRRL B-21262
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
In respect of those designations in which a European and/or Australia Patent is sought, during the pendency of the patent application, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC/Regulation 3.25 of Australia Statutory Rule 1991 No. 71).	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indication listed below will be submitted to the International Bureau Later (specify the general nature of the indications e.g. "Accession Number of Deposit")	

For receiving Office use only

<input type="checkbox"/> This sheet was received with the international application
Authorized officer

For International Bureau use only

<input type="checkbox"/> This sheet was received with the International Bureau on:
Authorized officer

## PATENT CLAIMS

1. A dyeing composition comprising an oxidation enzyme characterised in that the composition comprises:

- 5 1) one or more oxidation enzymes derived from a strain of the genus *Scytalidium*,  
2) one or more dye precursors, and  
optionally 3) one or more modifiers.

2. The dyeing composition according to claim 1, wherein the  
10 oxidation enzyme is derived from a strain of the genus *Scytalidium* laccase

3. The dyeing composition according to claim 2, wherein the laccase is derived from a strain of the species *Scytalidium thermophilum*.

15 4. The dyeing composition according to claims 2 and 3, wherein the laccase is neutral.

5. The dyeing composition according to claim 3, having the sequence shown in SEQ ID No 1.

20 6. The dyeing composition according to claim 5, wherein the sequence encoding the laccase is homologous to the SEQ ID NO 1.

7. The dyeing composition according to claim 6, wherein the sequence encoding the laccase is more than 80% homologous to SEQ ID NO 1.

8. The dyeing composition according to any of claims 1 to 7,  
25 comprising a dye precursor selected from the group comprising p-phenylene-diamine (PPD), p-toluylene-diamine, chloro-p-phenylenediamine, p-aminophenol, o-aminophenol and 3,4-diaminotoluene, 2-methyl-1,4-diaminobenzene, 4-methyl-o-phenylenediamine, 2-methoxy-p-phenylenediamine, 2-chloro-1,4-diamino-benzene,  
30 4-amino diphenylamine, 1-amino-4- $\beta$ -methoxyethylamino-benzene, 1-amino-4-bis-( $\beta$ -hydroxyethyl)-amonibenzene, 1-3-diamino-benzene, 2-methyl-1,3-diamino-benzene, 2,4-diaminotoluene, 2,6-diaminopyridine, 1-hydroxy-2-amino-benzene, 1-hydroxy-3-amino-benzene, 1-methyl-2-hydroxy-4-amino-benzene, 1-methyl-2-hydroxy-4- $\beta$ -hydroxyethylamino-benzene, 1-hydroxy-4-amino-benzene, 1-hydroxy-4-methylamino-benzene, 1-methoxy-2,4-diamino-benzene, 1-ethoxy-2,3-diamino-benzene, 1- $\beta$ -hydroxyethyloxy-2,4-diamino-

benzene, phenazines, such as 4,7-phenazinedicarboxylic acid, 2,7-phenazinedicarboxylic acid, 2-phenazinecarboxylic acid, 2,7-diaminophenazine, 2,8-diaminophenazine, 2,7-diamino-3,8-dimethoxyphenazine, 2,7-diamino-3-methoxyphenazine, 2,7-diamino-3-methoxyphenazine, 3-dimethyl 2,8-phenazinediamine, 2,2'-[(8-amino-7-methyl-2-phenazinyl)imino]bis-ethanol, 2,2'-[(8-amino-7-methoxy-2-phenazinyl)imino]bis-ethanol, 2,2'-[(8-amino-7-chloro-2-phenazinyl)imino]bis-ethanol, 2-[(8-amino-7-methyl-2-phenazinyl)amino]-ethanol, 2,2'-[(8-amino-2-phenazinyl)imino]bis-ethanol, 3-amino-7-(dimethylamino)-2,8-dimethyl-5-phenyl-chloride, 9-(diethylamino)-benzo[a]phenazine-1,5-diol, N-[8-(diethylamino)-2-phenazinyl]-methanesulfonamide, N-(8-methoxy-2-phenazinyl)-Methanesulfonamide, N,N,N',N'-tetramethyl-2,7-phenazinediamine, 3,7-dimethyl-2-phenazinamine, p-amino benzoic acids, such as p-amino benzoic acid ethyl, p-amino benzoic acid glycerid, p-amino benzoic acid isobutyl, p-dimethylamino benzoic acid amil, p-dimethylamino benzoic acid octyl, p-diethoxy amino benzoic amil, p-dipropoxy amino benzoic acid ethyl, acetylsalicylic acid, isatin derivatives, such as 2,3-diamino benzoic acid.

9. The dyeing composition according to claims 8, comprising a dye modifier selected from the group comprising m-phenylenediamine, 2,4-diaminoanisole, 1-hydroxynaphthalene ( $\alpha$ -naphthol), 1,4-dihydroxybenzene (hydroquinone), 1,5-dihydroxynaphthalene, 1,2-dihydroxybenzene (pyrocatechol), 1,3-dihydroxybenzene (resorcinol), 1,3-dihydroxy-2-methylbenzene, 1,3-dihydroxy-4-chlorobenzene (4-chlororesorcinol), 1,2,3-trihydroxybenzene, 1,2,4-trihydroxybenzene, 1,2,4-trihydroxy-5-methylbenzene, 1,2,4-trihydroxytoluene.

10. A method for dyeing comprising contacting a laccase derived from a strain of the genus *Scytalidium* with the keratinous fibres and at least one dye precursor in the presence or absence of at least one modifier for a period of time and under conditions sufficient to permit oxidation of the dye precursor into a coloured compound.

11. The method according to claim 10, wherein the dyeing is carried out at a pH in the range from 3.0 to 9.0, preferably 4.0 to 8.0, especially 6.0 to 8.0.

12. Use of an oxidation enzyme derived from a strain of the genus *Scytalidium* for oxidative dyeing keratinous fibres, in particular hair, fur, hide and wool.

13. The use according to claim 14, wherein the oxidation  
5 enzyme is derived from a strain of the species *Scytalidium thermophilum*.



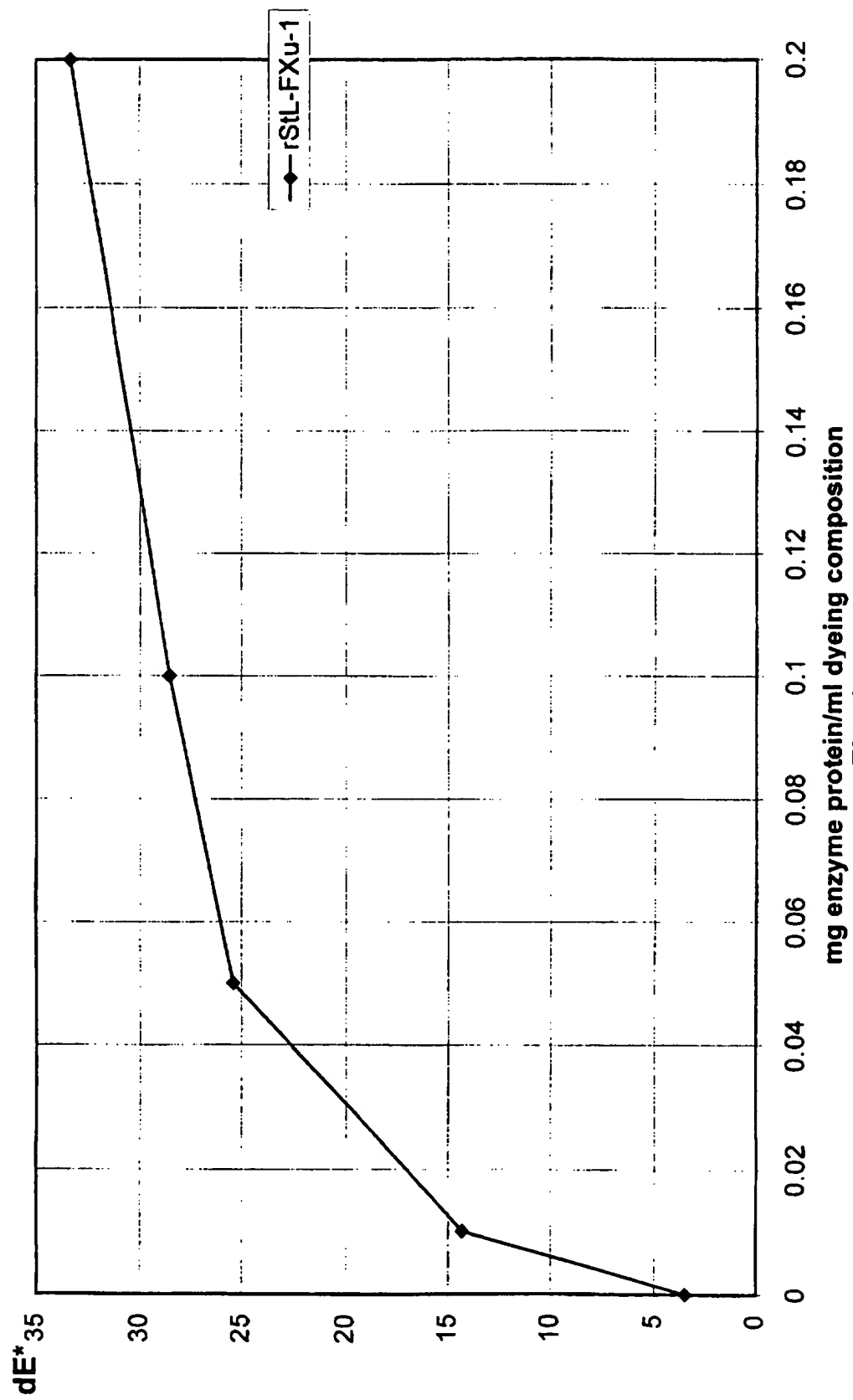


Fig. 1

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 96/00498

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C09B 67/00, A61K 7/13

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C09B, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9533837 A1 (NOVO NORDISK BIOTECH, INC.), 14 December 1995 (14.12.95), claims 28, 29; page 15, line 34 - page 16  --	1-13
P,A	WO 9533836 A1 (NOVO NORDISK BIOTECH, INC.), 14 December 1995 (14.12.95), claims 31-42; page 16, line 12 - page 17, line 27; page 34, line 20 - page 36  --	1-13
X	EP 0504005 A1 (PERMA SOCIETE ANONYME), 16 Sept 1992 (16.09.92)  --	1-13

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 96/00498

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 3251742 A (SAUL SOLOWAY), 17 May 1966 (17.05.66)  --	1-13
X	WO 9600290 A1 (NOVO NORDISK BIOTECH, INC.), 4 January 1996 (04.01.96), claims 37-48; page 48, line 25 - page 54, line 24  --	1-13
X	STN International, File CAPLUS, CAPLUS accession no. 1991:498981, Saruno, Rinjiro: "Hair- dyeing preparations containing melanin or other polyphenol pigments and manufacture of the pigments"; & JP, A2, 910403  --	1-13
X	STN International, File CAPLUS, CAPLUS accession no. 1995:974547, Chivukula, Muralikrishna et al: "Phenolic azo dye oxidation by laccase from Pyri- cularia oryzae"; & Appl. Environ. Microbiol. (1995), 61(12), 4374-77  --	1-13
A	DE 4314317 A1 (HENKEL KGAA), 3 November 1994 (03.11.94)  --	8
A	WO 9400100 A1 (L'OREAL), 6 January 1994 (06.01.94)  --	8
A	WO 9507988 A1 (NOVO NORDISK A/S), 23 March 1995 (23.03.95), claim 41  -- -----	1-13

# INTERNATIONAL SEARCH REPORT

Information on patent family members

03/02/97

International application No.

PCT/DK 96/00498

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9533837	14/12/95	AU-A- 2656695	04/01/96
WO-A1- 9533836	14/12/95	AU-A- 2656595	04/01/96
EP-A1- 0504005	16/09/92	AT-T- 121931	15/05/95
		CA-A- 2061826	09/09/92
		DE-D,T- 69202290	09/11/95
		ES-T- 2072720	16/07/95
		FR-A,B- 2673534	11/09/92
		JP-A- 6172145	21/06/94
US-A- 3251742	17/05/66	FR-A- 1363462	00/00/00
		GB-A- 993923	00/00/00
WO-A1- 9600290	04/01/96	AU-A- 2827895	19/01/96
DE-A1- 4314317	03/11/94	EP-A- 0695162	07/02/96
		JP-T- 8509478	08/10/96
		WO-A- 9424988	10/11/94
WO-A1- 9400100	06/01/94	DE-D,T- 69301464	05/06/96
		EP-A,B- 0645999	05/04/95
		FR-A,B- 2692782	31/12/93
		JP-T- 7508271	14/09/95
		US-A- 5538517	23/07/96
WO-A1- 9507988	23/03/95	AU-A- 7833694	03/04/95
		CA-A- 2171288	23/03/95
		CN-A- 1133067	09/10/96
		EP-A- 0719337	03/07/96
		FI-A- 961250	18/03/96
		US-A- 5480801	02/01/96